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Note

Growth pattern of stock cultures of five selected species of marine microalgae maintained under indoor controlled environment and under outdoor conditions

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ABSTRACT

Growth pattern of five species of microalgae *viz*., *Chaetoceros calcitrans*, *Dunaliella* sp., *Isochrysis galbana*, *Tetraselmis tetrahele* and *Nannochloropsis salina* were studied under indoor controlled conditions (at 23^0C) and under outdoor (at 28-30°C) conditions. The variations in ammonia and pH levels in the culture flasks over a period of 90 days were also studied. Results revealed that the pattern of growth of all five species of algae were significantly different ($p<0.05$) in the two different conditions. The results clearly showed that the growth of all the five species of algae were faster in outdoor conditions and outdoor cultures were able to maintain only for a maximum period of three months. Ammonia and pH levels recorded were higher in outdoor cultures which indicated higher physiological activities and growth. Ammonia and pH levels were found to increase gradually in all the cultures, upto 30 days under both conditions and subsequently found almost constant throughout the study. Even though ammonia levels were found to rise with increase in pH and temperature, it did not increase to a level that is detrimental to the microalgal cultures.

Keywords: Cell densities, Growth pattern, Microalgae, Stock culture, Temperature

Microalgae represent the largest group of microorganisms comprising of more than 80,000 species. Microalgae have important role in the aquatic ecosystems as primary producers and form one of the most important factors contributing to fish production. They are known to be energy capsules for the larval development of many commercially important molluscs, shrimps and fishes (Loosanoff and Davis, 1963; Ryther and Goldman, 1975). Cultured microalgae are being used as the sole food source in the intensive larviculture of shrimps, molluscs and many fish larvae especially in their early stages of life (Lambade and Mohamed, 2002). Cultivated microalgae form an integral part of hatchery production of many fishes and invertebrates. In India, there are more than 270 commercial shrimp hatcheries that produce penaeid shrimp larvae and all of them use microalgae or their products as the main larval feed (MPEDA, 2018).

Microalgal stock cultures are maintained in closely controlled laboratory conditions and mass cultures are generally carried out in large outdoor tanks. Batch culture method for mass culture of marine phytoplankton for shellfish hatcheries has been described by Gopinathan (1982). Though, batch culture is the relatively easy and most commonly used method of algal mass culture, its efficiency is poor and the cultures are prone to collapse easily. In order to avoid loss of cultures, stock of pure cultures are maintained in all algal culture laboratories.

Stock cultures of microalgae are usually maintained in controlled temperature conditions to regulate growth and also to avoid contamination. The physiology of microalgae is easily affected by many physicochemical factors such as temperature, salinity, light intensity, nutrient concentration and pH (Thomas, 1966) among which temperature has a major influence on the growth (Chen *et al*., 2012). However, in many seasonal shrimp/fish hatcheries air conditioned microalgal stock culture facilities are lacking and the main problem in these hatcheries are the uncertainty in algal production which may lead to heavy loss in larval production. Therefore this study was conducted as a part of the investigations to develop a protocol for the development and long term maintenance of outdoor algal stock cultures. The growth responses, cell densities, pH and Ammonia levels during different growth phases of five selected marine microalgal species were compared under indoor controlled conditions and under outdoor culture conditions.

Five species of marine microalgae *viz*., *Chaetoceros calcitrans* (CC), *Dunaliella* sp. (Dun), *Isochrysis galbana* (Iso), *Tetraselmis tetrahele* (TT) and *Nannochloropsis salina* (Nano) were used for the study. Pure cultures

obtained from the stock culture laboratory of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi were used for the study.

The experiment was conducted under two conditions *i.e*., in the indoor stock culture laboratory where controlled conditions are maintained (23^{0}C) and outdoor conditions at normal room temperature, where the temperature ranged from 28-30^{0}C (Table 1). Algal cultures were developed in Haufkin flasks (3 l capacity) filled with 1.5 l of filtered seawater having 35 ppt salinity. The flasks were cotton plugged and autoclaved. After 24 h, the flasks were enriched with f/2 media (Guillard and Ryther, 1962) and 10% inoculum was added to each flask to start cultures irrespective of cell density. Since no aeration was provided, all the flasks were shaken twice daily to avoid settling of cells. Indoor flasks were kept in air conditioned room (23^{0}C) on racks with fluorescent light (2700 Lux on the flask surface) at 12:12 light and dark photo cycle. Outdoor flasks were kept at room temperature with subdued sunlight that ranged from 2490 to 2860 lux and normal photoperiod. Light intensity was measured using a lux meter (Fisher Scientific, Envirometer 4 in 1). Cultures were observed once in two days to check for growth characteristics and contaminations. All cultures were maintained in triplicates for a period of 3 months.

For the estimation of standing crop, 1 ml sample was collected from each experimental flask and 4% formalin was added. The cell density was counted using a haemocytometer (0.1 mm deep Neubauer). For each sample, triplicate counting was done and the mean value was plotted for obtaining the growth curve. Growth rate was calculated as specific growth rate (μ). Increase in cell density per unit time was used for specific growth rate (μ) calculation in this study (Pirt, 1975).

$$\mu\ (day^{-1}) = \frac{\ln (X_1/X_0)}{t_1 - t_0}$$

where, X_0 and X_1 are the quantitative expressions of cell density, at start time (t_0) to the end time (t_1) between the selected time intervals during incubation.

Water samples from the culture flasks were centrifuged at 3000 rpm for 15 min and the clear supernatant (10 ml) was used f for estimation of ammonia. The samples were treated in an alkaline citrate medium with sodium hypochlorite and phenol in presence of sodium nitroprusside and the absorbance was measured in spectrophotometer (UV visible, Genesis-5, Thermo Spectronic) at 640 nm (CMFRI, 2001). Digital pH meter (Oakton 30) was used for measuring pH in the culture flasks. Both the parameters were estimated on a weekly basis till the end of the study.

The data were compared using one way ANOVA ($p<0.05$) in MS Excel and growth charts were plotted.

During the culture period, pattern of growth of all five algal species were found significantly different at the two different experimental conditions. Growth of marine microalgae is generally influenced by temperature, salinity, light intensity and nutrient concentrations (Thomas, 1996; Thomas and Sommerfeld, 1998) in which temperature is the major factor controlling the algal growth (Lund, 1949; Talling, 1955). Sorokin and Krauss (1962) also reported slow algal growth at lower temperatures, maximum growth at optimum temperature and the growth slows down or ceases at higher temperatures. Chen *et al*. (2012) reported that the microalgal growth rate is generally expected to increase with temperature and rapidly decline above the optimal temperature. According to Davison (1991), low temperatures usually reduce enzymatic activity, which may reflect reduction in growth rate. Results of the present experiments revealed that the growth phase was observed from 15-57 days under indoor controlled conditions whereas in outdoor conditions growth phase spanned from 14 to 35 days only indicating faster growth at higher temperatures (Fig.1a-e). During the present investigations, the lag phase was not prominent in almost all the cultures, except for *T. tetrahele* (indoor) and *N. salina* (outdoor). The former remained in lag phase for 5 days and latter for 3 days. The condition of the inoculum has a strong bearing on the duration of the lag phase. An inoculum taken from a healthy exponentially growing

Table 1. Range of water quality parameters of microalgae reared in indoor and outdoor conditions

	Temperature (°C)			pH						NH_3 (μg l^{-1})					
	OD			ID			OD			ID			OD		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
C. calcitrans	28.4	31.0	29.7±1.84	8.1	8.9	8.5±0.57	8	9.2	8.6±0.85	0.7	8	4.4±5.16	0.5	13	6.7±8.84
Dunaliella sp.	29.1	30.5	30.3±0.99	8	9	8.5±0.71	8	9.2	8.6±0.85	0.1	28	14±19.73	1.5	3.3	2.4±1.27
I. galbana	28.3	30.8	29.5±1.77	7.9	8.7	8.3±0.57	8	9.1	8.6±0.78	0.3	5	2.6±3.32	0.7	12	6.4±7.99
T. tetrahele	28.1	31.2	29.7±2.19	8	8.7	8.4±0.49	7.9	9	8.5±0.78	0.2	17	8.7±11.88	0.9	11	5.9±7.14
N. salina	29.0	30.9	29.9±1.34	8	9.2	8.6±0.85	8	9.4	8.7±0.91	0.7	6.6	3.7±4.17	0.4	9.1	4.7±6.15

OD-Out door culture, ID-Indoor cuture

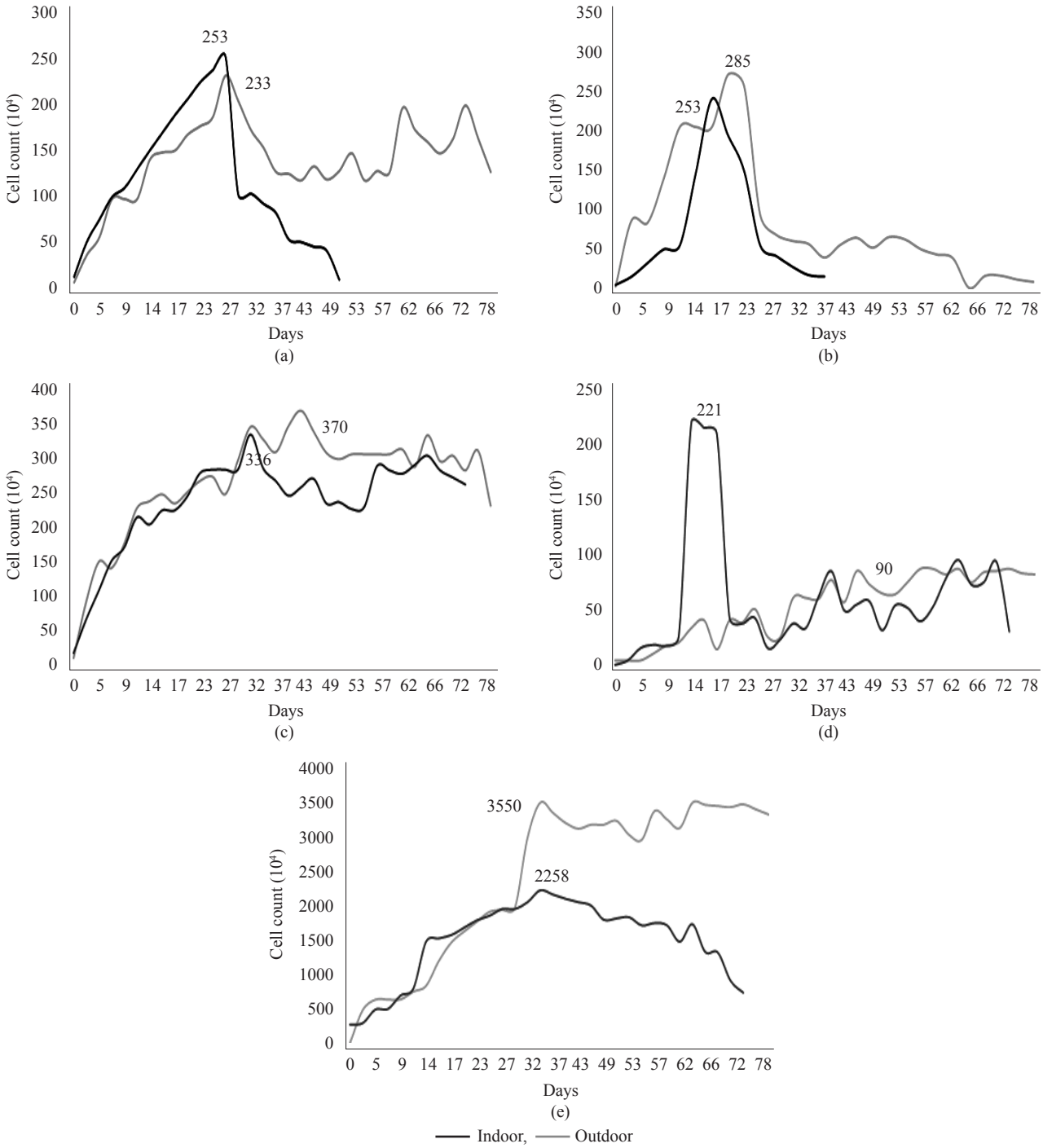

Fig. 1. Growth comparison of five microalgal species grown in indoor and outdoor conditions. (a) *C. calcitrans*, (b) *Dunaliella* sp. (c) *I. galbana,* (d) *T. tetrahele* and (e) *N. salina*

culture is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions of light, temperature and salinity (Spencer, 1954). The absence of lag phase in most of the cultures could be attributed to the good condition of the inocula used.

The growth rates were found higher in outdoor cultures compared to the indoor cultures, in the four out of the five microalgal species studied. *C. calcitrans* reached maximum biomass (2.33 x 10^6 cells ml^{-1}) on 27^{th} day in indoor and the maximum of 2.53 x 10^6 cells ml^{-1} on 25^{th} day in outdoor (Fig. 1a). Outdoor culture

also showed a sudden decline from the maximum count, compared to the indoor cultures. Increased growth rate in the outdoor conditions combined with the sudden decline just after the peak cell density (cell max) suggest that the stock culture of *C. calcitrans* can be maintained in outdoor conditions for only up to 26 days; whereas, under controlled low temperatures, the algal growth was steady and the culture could be extended even beyond the experimental period of three months. The results showed that under outdoor conditions, *C. calcitrans* needs to be subcultured preferably every two weeks for steady and healthy maintenance of stock cultures.

The culture duration of *Dunaliella* sp. was conspicuously shorter in outdoor (28 days) with a cell max of 2.64 x 10^6 cells ml^{-1} on 13th day indicating poor tolerance to higher temperatures. Whereas, the indoor cultures of *Dunaliella* sp. remained for 62 days (Fig. 1b) with slightly higher cell max (2.85 x 10^6 cells ml^{-1}). Even though the growth rate and the maximum cell densities were almost same in both the culture conditions, its sudden decline after the cell max in the outdoor condition can be an indication that this species also grow fast at higher temperatures. The results revealed that though outdoor stock culture of *Dunaliella* sp. is possible, it is not advisable as it is less consistent and there is all chances for frequent abrupt collapse after the cell max. Subculture has to be done every week in outdoor conditions, whereas under indoor low temperature conditions, it can be maintained for up to 62 days in good condition, though peak cell density was observed on 15th day.

I. galbana attained peak of 3.7 x 10^6 cells ml^{-1} on 43rd day in indoor conditions whereas in outdoor it took only 31 days to reach the cell max of 3.36 x 10^6 cells ml^{-1} (Fig. 1c). The culture of this algae remained in good conditions for the entire duration of the experiment in both indoor and outdoor. The growth rate, growth pattern as well as cell max of *I. galbana* was almost similar under both conditions indicating its wide temperature tolerance. Renaud *et al*. (2002) and Chen *et al*. (2012) reported that growth rate of *I. galbana* is higher at 25^{o}C. However, the present results revealed that temperature is not a crucial factor in the stock culture of *I. galbana*. It can be cultured up to 3 months under both conditions and subculture is suggested only once in a month for pure and healthy stock maintenance.

T. tetrahele showed remarkably slow growth rate under indoor conditions reaching a cell max of 0.96 x 10^6 cells ml^{-1} in 55 days (Fig. 1d). The indoor culture did not show much variation in cell densities throughout the culture period of 90 days after the initial lag phase. An average cell density of 0.62 x 10^6 cells ml^{-1} was maintained even at the termination of the experiment. Highest growth rate of *T. tetrahele* was noted in outdoor conditions in 14-17 days with cell density of 2.21 x 10^6 cells ml^{-1} but it lasted only for a short duration of 3 days. Then cell densities reduced and reached an average of 0.57 x 10^6 cells ml^{-1} and culture remained in good condition up to 2.5 months. In the case of *T. tetrahele,* growth was found stable under indoor conditions with slow growth rate. The culture of *T. tetrahele* remained in good condition in both indoor and outdoor culture condition up to a period of two months suggesting its hardy nature. From the results of the present study, it is suggested that in outdoor conditions, subculture should be done at least once in a month for a healthy stock maintenance whereas it may be needed only once in two or three months under indoor conditions.

For *N. salina,* peak density of 35.5 x 10^6 cells ml^{-1} was observed on 35th day under indoor conditions compared to 22.58 x 10^6 cells ml^{-1} in 34 days under outdoor conditions (Fig. 1e). The time taken to attain the peak density was almost same in both conditions; however cell density was higher in indoor cultures. The indoor cultures of *N. salina* remained in viable condition for a longer period (more than 3 months), even after the experimental period, whereas the outdoor culture collapsed after one month. Outdoor stock cultures are possible in this species and subculture is needed once in every three weeks.

Duration of growth phase in all these five species of algae lasted from 15-57 days in indoor and 14-35 days in outdoor conditions indicating noticeably faster growth in the higher temperatures (outdoor) as also reported by Sorokin and Krauss (1962) and Chen *et al*. (2012). *T. tetrahele* and *Dunaliella* sp. showed the shortest growth phases (14 days) in outdoor conditions. Whereas, shortest growth phase (15 days) in the indoor cultures was noticed in *Dunaliella* sp. However, prolonged growth is observed in *T. tetrahele.* The duration of growth phases of *C. calcitrans, N. salina* and *Dunaliella* sp. were found almost similar in both the conditions (27, 35 and 43 days respectively). All the outdoor cultures were found crashed before the end of the experiment. Outdoor cultures of *C. calcitrans* and *Dunaliella* sp. completely crashed on 21st and 29th days respectively. Whereas, *T. tetrahele, I. galbana* and *N. salina* crashed completely only on 76th day in outdoor conditions. In indoor conditions, all the stock cultures except *Dunaliella* sp. remained in good condition till the end of the experiment. The highest cell density was observed in *N. salina* (35.5 x 10^6 cells ml^{-1} in indoor and 22.5x10^6 cells ml^{-1} in outdoor) and the lowest was in *T. tetrahele* (0.9 x 10^6 cells ml^{-1} in indoor and 2.21x10^6 cells ml^{-1} in outdoor). The cell densities were generally found more in indoor conditions but in *T. tetrahele* the density was found considerably high in

outdoor cultures. A slight increase in cell density was also observed in the outdoor cultures of *C. calcitrans*.

In both experimental conditions, the initial pH ranged from 7.9-8.1 (Table 1). Ammonia and pH levels were found higher in outdoor cultures during the study period compared to indoor cultures suggesting its higher physiological activities and growth. Ammonia and pH content was found increased in all the cultures, upto 30 days under both conditions and then found almost constant. Many reports are there indicating that the pH values changes significantly in marine systems despite the strong buffering capacity of the carbonates in seawater (Marshal and Orr, 1948; Frithsen *et al*., 1985; Pegler and Kempe, 1988). In general, changes in pH levels in marine systems appear to correlate with changes in temperature, dissolved oxygen and phytoplankton production. In this study pH values were comparatively higher in outdoor cultures (Fig. 2a-b). The removal of CO_2 by photosynthetic uptake may also lead to an increase in pH in the outdoor system (Paul and John, 2005). This could be one of the reasons for increase in pH during the first month when cultures were in growth phase. As the culture entered stationary phase, removal of CO_2 is reduced due to slow rate of photosynthesis and subsequent reduction in pH. Celia and Durbin (1994) observed consistent decline of growth and photosynthesis at pH levels >8.8. Pruder and Bolton (1979) also reported interaction of pH and CO_2 resulted in reduced growth of *Thalassiosira pseudonana*.

In both the experimental conditions, the highest value of ammonia was noticed in the culture of *Dunaliella* (28.02 and 15.28 µg l^{-1}) in indoor and outdoor respectively (Fig. 3a-b). This may be a reason for the comparatively short culture duration of this species both in outdoor and indoor conditions. Though, the ammonia level was less in outdoor cultures than in the indoor conditions, the cumulative effects of high levels ammonia and elevated

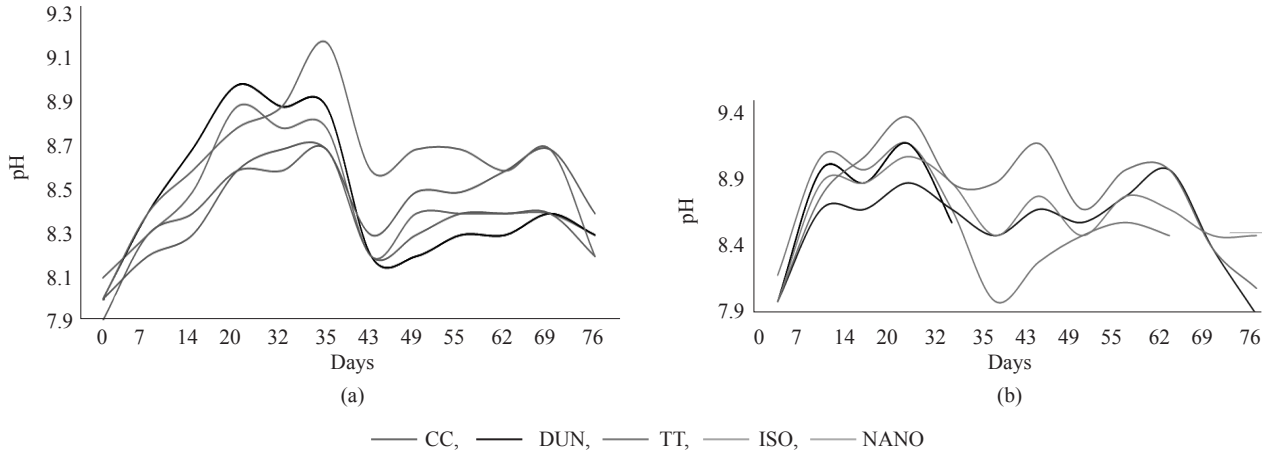

Fig. 2. Variations of pH levels of microalgae cultured in indoor and outdoor conditions. (a) pH - Indoor, (b) pH - Outdoor

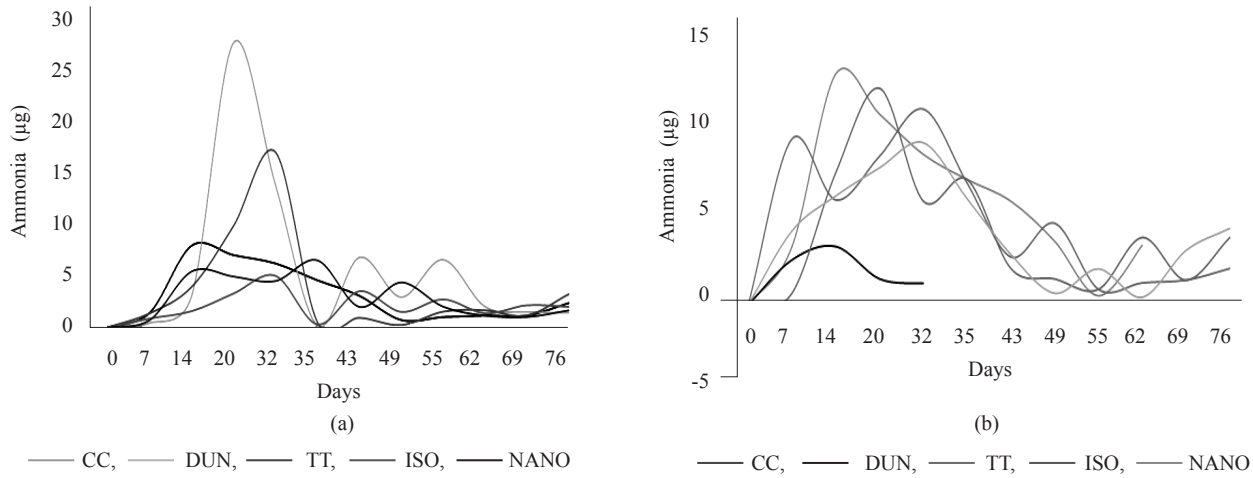

Fig. 3. Variations of ammonia levels of microalgae cultured in indoor and outdoor conditions. (a) Ammonia - Indoor, (b) Ammonia - Outdoor

temperature might have resulted in the early collapse of the outdoor cultures of *Dunaliella*. The lowest ammonia levels were noticed in *N. salina* (4.56 µg l^{-1}) in indoor and *I. galbana* (5.79 µg l^{-1}) in outdoor. The highest pH was reported in *N. salina* under both the conditions (9.2 in indoor and 9.4 in outdoor). After 30-35 days, pH level was found to decrease and were found in the range of 8.2-8.6 in indoor and 7.9-8.9 in outdoor.

The increase in pH of the medium by photosynthetic uptake of CO_2 has a direct link with the increase in NH_3 concentration (Chen *et al*., 2012). It was also observed that the ammonia level increased with increase in pH and temperature. Admiraal (1977) showed that concentrations of 0.247 mg l^{-1} ammonia retarded growth of seven species of benthic diatoms. In contrast, lower levels of ammonia is regarded as one of the main nitrogen source for microalgae (Abe *et al*., 2002; Soletto *et al*., 2005; Converti *et al*., 2006). Hii *et al.* (2011) reported faster growth in *Nannochloropsis* sp. in presence of ammonia. Thus the potential effects of ammonia on phytoplankton growth can be either stimulatory or inhibitory with species specific responses to different ranges of ammonia concentrations (Livingston, 2002). In this study, even though ammonia levels were found to rise with the increase in pH and temperature, it did not increase to a level that is detrimental to the microalgal stock cultures. From this study it is inferred that stock cultures of all these five species of microalgae can be done in outdoor conditions by following correct protocols.

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